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The Collection and Preparation of Human Blood Plasma or Serum for Trace Element Analysis

J. Versieck

University of Ghent, De Pintelaan 185, B-9000 Ghent, Belgium

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Trace element concentrations in blood plasma or serum have been assayed by numerous investigators using a variety of analytical techniques. For several elements, figures obtained in different centers are widely disparate. Impressive evidence has accumulated that a great deal of the inconsistencies should be ascribed to unsuspected contamination of the samples with exogenous material during their collection and preparation. In this paper, a number of potential sources of extraneous additions are indicated. Methods for controlling contamination are also briefly discussed.

Key words: blood collection devices; clean room conditions; high-purity reagents; sample containers; sample contamination.

1. Introduction

Circulating blood consists of formed elements (red blood cells, white blood cells, and platelets) suspended in a fluid (native plasma). Unless an anticoagulant is added, normal blood withdrawn from the circulation forms a clot due to the polymerization of fibrinogen to fibrin. On standing, the clot retracts, expressing serum which differs from plasma chiefly in that it contains no fibrinogen. When an anticoagulant (heparin, potassium oxalate, sodium citrate, or another) is added, clotting is delayed or prevented. So, plasma can be separated by centrifugation. In this way, different types of cells and platelets can also be isolated for investigation.

Blood and its constituents are frequently submitted to clinical and experimental laboratories for chemical analyses. Both because of their established importance to life and their ready accessibility, these matrices also occupied the attention of numerous trace element investigators. Particularly plasma and serum have been the subject of intensive search.

Many of the chemical substances in whole blood are present in unequal concentrations in the different fractions. Whole blood analyses give only overall values of potentially widely differing levels. Most trace elements occur at a higher concentration in packed blood cells (in ng or μg/g wet weight) than in plasma or serum (in ng or μg/mL): for selenium, the ratio is about 1.2, for cesium 6.5, for zinc 10.5, for rubidium 25.2, and for manganese 26.3. However, there are exceptions: for copper, the ratio is about 0.62 and for molybdenum 0.41 [1].[1]

A survey of published data on trace element levels in human blood plasma or serum shows that widely divergent values were measured in different laboratories. Certainly, a few sources of physiological variations are well documented [2]. However, solid experimental evidence has been accumulated that shows that much of the existing controversy should be ascribed to poor analysis or to inadequate sample collection and preparation [3].

About the Author: J. Versieck is with the Department of Internal Medicine, Division of Gastroenterology, University Hospital, University of Ghent.

[1] Figures in brackets indicate literature references.

There are a number of factors which threaten sample integrity—undoubtedly, unsuspected contamination with exogenous material is the most important.

2. Methods

Different approaches have been employed to assess the impact of unwanted extraneous additions on the accuracy and precision of trace element measurements in human blood plasma or serum

Most published information is based on indirect measurements. Indeed, most investigators suspecting extraneous additions changed their sampling procedures until the lowest values were obtained and estimated the errors from the difference between their original high values and final low values.

At the University of Ghent, this approach was also used but, in addition, a method was developed to estimate the errors more directly. Using neutron activated instruments and other materials with which a sample may come into contact, several sample collecting and handling steps were reproduced in vitro with the underlying idea that, in these experimental conditions, additions from the devices to the samples should be reflected by traces of radioactivity in the samples. In the first place, this approach allows a rapid identification of potential sources of contamination. In addition, by using suitable irradiation conditions and post-irradiation measurements, it is possible to estimate the unwanted additions of a number of elements simultaneously. Indeed, the photopeaks detected in the samples may, of course, be converted into quantitative values in exactly the same way as in routine neutron activation analysis. The technique has been described in detail elsewhere [4].

3. Review and Discussion

The vital importance of adequate sampling was recognized by leading authorities a considerable time ago, yet it would appear that the level of practice among many investigators left much to be desired. Thus, the warning issued by Thiers in 1957 [5] that "unless the complete history of any sample is known with certainty, the analyst is well advised not to spend his time analyzing it" was largely ignored for several years.

Cotzias and his co-workers again attracted the attention to the problem [6,7]. In 1961, these investigators reported a mean plasma manganese concentration of 2.69 ng/mL which, five years later, they acknowledged to be unreliable because a careful search revealed that a systematic contamination with exogenous metal had persisted in their first study. At that moment, they came to the conclusion that the true value was 0.587 ± 0.183 ng/mL—a value nearly five times lower than the first. Shortly after, Davies and his colleagues [8] showed that routine plasma zinc determinations, whether fasting or at random, are of no value unless a few simple but stringent precautions are observed. They noted that a series of zinc estimations in plasma from blood samples of apparently healthy individuals, taken without special precautions, showed an aberrantly high mean (about 1.33 μg/mL) and an erroneously large spread (from about 0.86 μg/mL up to about 1.66 μg/mL) when compared to the true values obtained under adequately controlled conditions (mean of 0.95 μg/mL in men and 0.96 μg/mL in women; range from 0.76 μg/mL to 1.25 μg/mL for either sex).

In the author's laboratory, systematic studies were initiated at the end of the 1960s [4]. A general survey of the results, obtained in the course of the years, was published in Talanta in 1982 [9].

Using the direct approach, very important additions were observed in blood samples collected with irradiated, disposable, steel needles. In a series of experiments, the most striking contaminations were invariably found in the first 20-mL samples. Thus, the iron contamination in the first 20-mL sample amounted to about 15% of the expected, intrinsic level of the element in plasma or serum and to about 2% in subsequent 20-mL samples. The manganese contamination in the first 20-mL samples varied from about 13 to 77% and in the third or fourth 20-mL samples from about 2 to 10%. The transfers of cobalt and, more particularly, of chromium and nickel were even more important as they may equal and even largely exceed the intrinsic levels of the elements in human serum. For chromium, e.g., additions varying from 90 ng/mL in the first 20-mL sample to 10 ng/mL in the third 20-mL sample were observed whereas the true value in human serum has been estimated to be about 0.15 ng/mL. The additions of scandium, silver, tin, antimony, and gold are difficult to interpret because, in several instances, only upper limits could be established and because the uncertainty surrounding the plasma or serum levels of these elements continues. Of the additions examined, only those of copper and zinc turned out to be negligible [9].

To avoid these serious artifacts, it was decided to take blood samples with a polypropylene catheter (Intranule®, Vygon). Studies showed that the manganese additions were considerably reduced—the largest errors that were observed varied from 3 to 4%.

The transfers of manganese and copper to serum samples stored in polyethylene containers were also studied. Some of them were not cleaned, others were briefly rinsed with bidistilled water. The absolute amounts of the additions of both elements were found to be roughly of the same order of magnitude (mean values, non-

cleaned containers—manganese: 0.57 ng/mL, copper: 0.96 ng/mL; rinsed containers—manganese: 0.084 ng/mL, copper: 0.27 ng/mL). It is evident, however, that the significance is widely different: indeed, when compared to the normal mean plasma or serum levels of the elements (manganese: about 0.55 ng/mL, copper: about 1.0 μg/mL), the observed copper additions are negligible whereas, on the contrary, the observed manganese additions are very significant! These data also illustrate the vital importance of cleaning all containers with extreme care. If they are only rinsed with bi-distilled water, for manganese, errors of up to 15 or 20% may easily persist.

It may be argued that, in these experimental conditions, irradiation damage to the instruments may have increased the extraneous additions, particularly when instruments, like venipuncture needles, were irradiated for a long time at high neutron fluxes, e.g., for the study of chromium, iron, cobalt, and nickel additions (irradiation: five days, neutron flux: 10^{14} n·cm^{-2}·s^{-1}). Personal results obtained using the indirect approach, however, also prove that the artifacts may be extremely important.

For example, the concentration of manganese, copper, and zinc was assayed by neutron activation analysis in duplicate serum samples of 12 patients with minor ophthalmologic disorders but without apparent signs of other health problems. Initially, the mean ± standard deviation for manganese was found to be 6.7±6.6 ng/mL or 6.9±7.3 ng/mL. The variability between duplicate samples appeared to be very important ($\sqrt{\Sigma d^2/2N}$=5.7 ng/mL; d=difference between the two results in a duplicate determination, N=number of duplicate determinations performed). After the sampling procedure was substantially refined (use of a plastic catheter for venipuncture; thoroughly cleaned, high-purity synthetic quartz tubes and conventional polyethylene containers for collection, storage, lyophilization, and irradiation; transport of samples under carefully secluded conditions; working under clean room conditions), employing exactly the same radioanalytical technique, values turned out to be 0.63±0.10 ng/mL or 0.64±0.14 ng/mL in a comparable series of subjects. In this second series, the variability between duplicate samples appeared to be minimal ($\sqrt{\Sigma d^2/2N}$=0.074 ng/mL [9]. It is interesting to note that the results for copper and zinc in both series were nearly identical. This illustrates that a sampling procedure may be adequate for analyses at the μg/mL (serum copper and zinc) yet grossly deficient for determinations at the ng/mL level (serum manganese).

Table 1 shows another example. In the upper part, it catalogues the values obtained by neutron activation analysis for manganese, copper, and zinc in nine 1-mL serum samples transferred in the liquid state with a digital dispenser (manufactured by Hamilton; PTFE tubing system) into previously cleaned, conventional polyethylene containers for lyophilization and irradiation. Before use, the system was flushed with quartz-bidistilled water. In the lower part, it lists the results found in nine other samples that were first lyophilized and then transferred with a carefully cleaned, small quartz spoon (Spectrosil®, Thermal Syndicate) into thoroughly cleaned, identical conventional polyethylene containers for irradiation, as done in the laboratory for many years. It is evident that one misstep may heavily distort the figures for a low-level trace element like manganese (concentrations measured in samples transferred with dispenser—mean value: 4.83 ng/mL, range: 0.61—16.83 ng/mL; in samples transferred with quartz spoon—mean value: 0.72 ng/mL, range:

Table 1. Manganese, copper, and zinc levels measured in nine serum samples transferred with a digital dispenser (manufactured by Hamilton; PTFE tubing system) and in nine others transferred after lyophilization with a thoroughly cleaned, high-purity quartz spoon. All measurements were done by exactly the same radiochemical technique.

Samples transferred with Digital Dispenser	Mn (ng/mL)	Cu (μg/mL)	Zn (μg/mL)
1	0.71	1.03	0.84
2	1.38	1.14	0.94
3	0.61	0.98	0.88
4	2.39	1.01	0.99
5	8.56	1.07	0.99
6	5.66	1.07	0.90
7	16.83	1.01	0.91
8	3.92	1.10	0.88
9	3.39	0.97	0.84
Mean	4.83	1.04	0.91
Range	0.61–16.83	0.98–1.14	0.84–0.99

Samples transferred with Quartz Spoon	Mn (ng/mL)	Cu (μg/mL)	Zn (μg/mL)
1	0.74	1.11	1.05
2	0.56	1.01	0.98
3	0.89	1.07	0.98
4	0.64	1.01	0.90
5	0.66	1.03	0.96
6	0.74	1.07	1.02
7	0.70	1.03	0.94
8	0.82	1.04	0.97
9	0.69	1.08	0.84
Mean	0.72	1.05	0.96
Range	0.56–0.89	1.01–1.11	0.84–1.05

0.56–0.89 ng/mL) although the figures for copper and zinc are not markedly affected.

Scattered throughout the literature are reports of other investigators who cautioned against the errors from inadvertent sample contamination in biomedical trace element investigations.

Published data on contamination from venipuncture needles are scarce. Kumpulainen and his colleagues [10] compared chromium concentrations in serum from blood samples collected with conventional steel needles and with plastic catheters (Venflon®, Viggo): in the first, they found a mean value of 0.43 ng/mL, and in the second, of 0.12 ng/mL. Sunderman and his co-workers [11] measured nickel concentrations in serum samples (21 healthy adults) from blood collected from one arm with 22 gauge "Monoject" needles (about 71.1% of iron, 17.4% of chromium, 9.1% of nickel, and small amounts of other elements, e.g., 1.6% of manganese, 0.43% of molybdenum, 0.12% of colbalt, and 0.047% of tungsten) [12] and from the other arm with polyethylene intravenous cannulas. In the first case, the levels averaged 0.74±0.25 ng/mL, in the second 0.37±0.18 ng/mL. The mean difference between the paired nickel concentrations was found to be 0.38±0.23 ng/mL.

The risk of obtaining misleading serum zinc values because of extraneous additions from the rubber stoppers of evacuated blood collection tubes of various kinds (Labtube®, Abbot; Vacutainer®, Becton-Dickinson; Venoject®, Kimble-Terumo) has been identified by numerous investigators [13–18]. Thus, Williams [18] determined iron, copper, and zinc in serum or plasma collected from normal volunteers in plain, acid-washed test tubes on the one hand and in plain, lead-free, and heparinized Vacutainers® on the other. The mean values for both iron and copper were perfectly comparable, regardless of the tubes used. In contrast, mean zinc values were consistently higher when plain, lead-free and heparinized Vacutainers® were used (respectively 1.94±0.064 μg/mL, 2.50±0.127 μg/mL, and 1.84±0.072 μg/mL versus 1.05±0.023 μg/mL—value measured in serum from blood collected in plain, acid-washed, glass test tubes). These observations prompted Becton-Dickinson to develop a new type of stopper for trace element studies (tube with minimal trace element content, royal blue stopper). All studies showed that the contamination of the samples with zinc was strongly reduced [9,17,18]: thus, using this tube, Williams measured a serum zinc value of 1.16±0.023 μg/mL [18]. Our investigations,however, indicate that it offers only an incomplete solution. Indeed, considerable contamination with manganese was found to persist: additions varied from 0.044 to 0.292 ng/mL and from 0.064 to 0.918 ng/mL in samples that remained in contact with the stopper for respectively 30 and 120 minutes, whereas the true mean serum manganese concentration in healthy adults is now generally believed to be about 0.55 or 0.60 ng/mL [9,19]. Furthermore, its reliability in the assay of other low-level trace elements such as vanadium, chromium, cobalt, arsenic, and molybdenum remains to be established.

In general, the sample container is one of the potentially largest sources of sample contamination. Much of the analytical accuracy will depend upon the choice of the material and the method of cleaning. For this important issue, the reader is referred to the work of Moody and Lindstrom [20] who examined the levels of impurities in various plastics (conventional polyethylene, linear polyethylene, polypropylene, several types of Teflon®, and a number of other materials) and made recommendations for cleaning methods.

The paper of Reimold and Besch [17] also contains information on other potential sources of contamination with zinc (plastic tubes, Parafilm®, wooden applicator sticks, chemical reagents, laboratory tissues like Kimwipes® and Kleenex® for wiping pipettes and glassware, filter paper, etc.). Detailed information on impurities in chemical reagents has been published by Murphy [21] and Zief and Micholotti [22]. Techniques for preparing exceptionally high-purity reagents, which are especially valuable for the stabilization of trace metals in solution and for the dilution of samples, have been described by Kuehner et al. [23], Mitchell [24], and Moody and Beary [25].

Heydorn and associates [26,27] drew attention to the potential errors caused by airborne particulate matter,usually referred to as dust, in serum samples intended for manganese determinations. An analysis of 11 duplicate serum samples, taken with great care to avoid contact with materials likely to contaminate the samples with traces of the element, revealed the presence of unknown sources of variation. The precision of the analytical technique being well established, it was concluded that the duplicates were not identical but caused an additional, estimated standard error of about 0.35 ng/mL. Another set of samples was obtained by the same technique but under more secluded conditions, keeping them covered essentially all the time. The resulting measurements clearly showed that a highly significant reduction of variation between duplicate results was obtained (standard error of 0.04 ng/mL) as a consequence of shielding the samples from airborne contamination.

In addition, the potential for trace element contamination from air particulates has been examined by many other investigators [21,28–32]. Leading experts in trace and ultratrace analysis consider a clean laboratory to be an essential requirement to reduce this source of contamination. It can be assumed that plasma or serum

samples for the determination of elements in the μg/mL range, e.g., iron, copper, zinc, and a few others, can be adequately handled in ordinary analytical and clinical laboratories—at least when some basic technical rules and principles are strictly observed. On the other hand, when concentrations go down to the ng or sub-ng/mL range, e.g., aluminum, vanadium, chromium, manganese, cobalt, nickel, arsenic, molybdenum, and others, the whole prospect changes and all routine cleanliness or precaution practices become insufficient. In those cases, the key to successful measurements is found to be in the control of the analytical blank and a clean laboratory environment is one of the major tools available to the researcher. In 1982, the design of the clean laboratories for trace element analysis at the National Bureau of Standards has been authoritatively described by Moody [33].

The foregoing survey will have made it clear that unsuspected extraneous additions during the collection and preparation of plasma or serum samples may have devastating effects on the results of trace element assays. All potential sources need the greatest attention from the investigator. Rigid control of one source is not sufficient. Thus, controlling the environmental blank caused by air particulates, however important it may be, will be of little value if other sources of contamination such as collection devices, containers, and reagents are out of control. Scrupulous efforts must be undertaken to eliminate deficiencies at all stages.

4. Conclusions

Sample collection and preparation have been relatively neglected areas in trace element research. Advances in contamination control made it clear that they may be the origin of more serious errors than any other step in the analytical process. This is particularly true in the case of blood plasma or serum because the intrinsic levels of many elements are extremely low [3,9–11,34–38]. Control of contamination holds the key to further progress in accuracy and will permit the exploitation of the full sensitivity and specificity range of the existing analytical procedures in practical studies. Hopefully, this paper will contribute to the ultimate disappearance from the literature of papers reporting reference values or variations in physiological and pathological conditions based on estimations of trace element levels in obviously contaminated samples!

5. References

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